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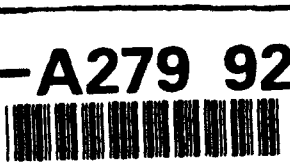

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Corticotropin-releasing factor increased $[Ca^{2+}]_i$ via rec receptor-mediated Ca^{2+} channels in human epidermoid A-431 cells

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Abstract

Corticotropin-releasing factor (CRF) has been shown to attenuate vascular leakage in injured skin, mucous membrane, muscle, and brain. Calcium is thought to play an important role in many of the physiological responses to CRF, but there has been little characterization of how calcium is involved in process by which CRF protects damaged tissues. The goal of this study was to characterize changes in cytosolic free calcium concentrations ($[Ca^{2+}]_i$) in human epidermoid A-431 cells exposed to human/rat-CRF and to investigate the mechanisms by which these changes occur. The resting $[Ca^{2+}]_i$ in normal cells at 37°C was 66 ± 4 nM ($n = 32$). When cells were treated with CRF, $[Ca^{2+}]_i$ increased immediately. The increase depended on CRF concentration, with a median effective concentration of 11 pM. This increase in $[Ca^{2+}]_i$ depended on external Ca^{2+} but not Na^+, Mg^{2+}, or K^+. La^{3+} (10 μM) and Co^{2+} (10 μM) inhibited the CRF-induced $[Ca^{2+}]_i$ increase, whereas verapamil and nifedipine tested at concentrations up to 1 mM did not. α-Helical CRF-(9–41), a synthetic CRF receptor antagonist, and pertussis toxin blocked the increase in $[Ca^{2+}]_i$ induced by CRF, which suggests that the entry of extracellular Ca^{2+} is mediated by receptor-operated Ca^{2+} channels coupled with pertussis toxin-sensitive G proteins. Although 420 pM CRF stimulated an immediate increase in $[Ca^{2+}]_i$, inositol trisphosphate and cellular cAMP levels did not change within 1 min either in the presence or absence of external Ca^{2+}. U-73122 (an inhibitor of inositol trisphosphate production), amiloride and benzamil (inhibitors of the Na^+/Ca^{2+} exchanger) also did not block the increase in $[Ca^{2+}]_i$ induced by CRF. CRF also increased $[Ca^{2+}]_i$ in cells treated with TMB-8 or ryanodine, inhibitors of intracellular Ca^{2+} mobilization. The results suggest that CRF stimulates a Ca^{2+} influx through CRF receptor-operated Ca^{2+} channels.

Key words: Ca^{2+}; Corticotropin-releasing factor; Ca^{2+} channel; Pertussis toxin; Epithelium

1. Introduction

The endocrine function of corticotropin-releasing factor (CRF) to stimulate adrenocorticotropin (ACTH) release is well-characterized, and its distribution throughout the body has been demonstrated. Using immunohistological techniques, CRF-like substances have been detected in many systemic organs including liver, spleen, pancreas, stomach, duodenum, and adrenal medulla (Petrusz et al., 1984). CRF is also present in hypothalamus and other parts of brain (Merchenthaler, 1984). The CRF levels in hypohyseal portal blood and systemic plasma in human are 400 and 75 pg/ml, respectively (Plotsky, 1985). CRF is present in the final stages of human pregnancy at a level about 20 times the normal level (Linton et al., 1990a,b; Wolfe et al., 1988).

The exposure of tissues to exogenous CRF produces a variety of effects, including a decreased heart rate and blood pressure (Kiang and Wei, 1986) and a reduction in the detrimental effects of several kinds of injuries (Kiang and Wei, 1987; Wei and Kiang, 1987; Wei et al., 1988; Serda and Wei, 1991, 1992; Wei and Gao, 1991).

The mechanism by which CRF reduces the edema and protein extravasation characteristic of such injuries is not known. It is possible that changes in cytosolic free calcium ($[Ca^{2+}]_i$) play an essential role in CRF protection because other CRF actions have been shown to involve Ca^{2+}. For example, in human ACTH-secreting pituitary adenoma cells and normal rat small ovoid

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corticotrophs, Guerineau et al. (1991) found that 100 nM CRF increased $[Ca^{2+}]_i$ by opening both L- and T-type voltage-gated Ca^{2+} channels. In corticotroph-derived AtT-20 cells, the CRF-induced increase in c-*fos* mRNA is reduced by blocking Ca^{2+} entry or by treatment with calmodulin inhibitors (Boutillier et al., 1991). Furthermore, ACTH release stimulated by CRF is reduced in the absence of external Ca^{2+}, by depletion of intracellular Ca^{2+} stores, or by treatment with calmodulin inhibitors (Won and Orth, 1990).

It has been shown that the epidermal necrosis produced by heat injury in rat paw skin (Wei et al., 1987) and human epidermoid A-431 cells (Kiang et al., 1988), a non-endocrine cell line, is reduced by pretreatment with CRF. Because Ca^{2+} homeostasis in A-431 cells has been extensively described (Kiang, 1991; Kiang et al., 1992), we used these cells to characterize the effect of CRF on $[Ca^{2+}]_i$ and investigate the underlying mechanisms of action. The results of our experiments show that CRF treatment caused an increase in $[Ca^{2+}]_i$ that was antagonized by α-helical CRF-(9–41), a CRF receptor antagonist.

2. Materials and methods

2.1. Cell culture

Human epidermoid carcinoma A-431 cells (American Type Culture Collection, Rockville, MD) were grown on glass cover slips (9 × 35 mm, Clay Adams, Lincoln Park, NJ) incubated at 37°C in a 5% CO_2 atmosphere. The tissue culture medium was Dulbecco's modified Eagle medium supplemented with 0.03% glutamine, 4.5 g/l glucose, 25 mM Hepes, 10% fetal bovine serum, 50 μg/ml penicillin, and 50 U/ml streptomycin (Gibco Laboratories, Grand Island, NY). Cells were fed every 3–4 days. Cells from passage 28–45 were used for experiments.

2.2. Measurements

Intracellular Ca^{2+} measurements

Confluent monolayers of cells were loaded with 5 μM fura-2AM plus 0.2% pluronic F-127 (to make cells more permeable to the probe) at 37°C for 60 min. Cells were washed with Na^+ Hanks' solution (in mM: 145 NaCl, 4.6 KCl, 1.2 $MgCl_2$, 1.6 $CaCl_2$, and 10 HEPES, pH 7.4 at 24°C) before fluorescence measurements. The method to determine $[Ca^{2+}]_i$ has been described previously (Grynkiewicz et al., 1985; Kiang, 1991). Briefly, the confluent monolayer of cells was placed in a thermostatically controlled cuvette that was kept at a constant temperature of 37°C. The fluorescence signal was measured with the emission wavelength set at 510 nm and dual excitation at 340 nm and 380 nm (slit width 4 nm) with a PTI DeltaScan spectrofluorometer (Photon Technology International, South Brunswick, NJ). Autofluorescence from cells not loaded with the dye was in the range of 3000–4000 photons/s and was subtracted from the fura-2 signal. Fura-2 leaked out of A-431 cells at a rate of 0.38 ± 0.01%/min ($n = 3$) at 37 °C. To minimize any contribution to the fluorescence signal resulting from dye in the medium, cells were washed thoroughly in Hanks' solution before they were transferred to a cuvette to measure $[Ca^{2+}]$. $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al. (1985).

Inositol trisphosphates measurements

Inositol trisphosphates ($InsP_3$) were measured as described by Berridge (1983) and my laboratory (Kiang and McClain, 1993). In brief, cells were grown in 6-well plates and incubated with *myo*-[^{3}H]inositol (2 μCi/ml, 0.22 nmol/ml, Dupont/NEN, Boston, MA) for 24 h. Cells were washed twice with Na^+ Hanks' solution then incubated with CRF for 5 s, 1, 5, or 10 min. The reaction was stopped by adding 3 ml ice-cold 4.5% $HClO_4$:Na^+ Hanks' solution (2:1, v/v). Each plate was placed on ice for 30 min, and the cells were removed by scraping. Cells were pelleted by centrifugation (750 × *g*) and the supernatants were collected. The pH of the supernatants was adjusted to pH 8.0 with KOH before storing at −70°C. An aliquot (100 μl) of supernatant, which contained all of the [^{3}H]inositol and [^{3}H]polyphosphoinositol metabolites, was counted to determine total radioactivity in the cells. [^{3}H]$InsP_3$ was eluted by 100 mM formic acid in 1.0 M ammonium formate (38 ml) from a Dowex AG 1-× 8 resin column. Radioactivity was determined by mixing 1 ml of the eluent with 10 ml of aquasol scintillation cocktail and counted with a scintillation counter. The amount of $InsP_3$ was then expressed as a percentage of total counts per minute in each well.

cAMP measurements

Confluent monolayers of cells (2×10^6/35 mm culture dish) were washed twice with PBS before incubating with PBS for 1 h at 37°C. Incubation was then continued with 3-isobutyl-1-methylxanthine (IBMX, final concentration 1 mM) for 30 min at 37°C, after which the medium was removed. Na^+ Hanks' solution containing CRF was added into the culture dish for 5 s, 1, 5 or 10 min. The medium was discarded, and 1 ml of 0.1 M HCl was added to each culture dish to stop the reaction by killing the cells. The culture dishes were maintained at 4°C for 1–2 h before transferring the suspensions to test tubes. The samples were boiled for 10 min and the precipitates removed by centrifugation at 2000 × *g* for 10 min. The supernatants were stored at −20°C until the cAMP measurement.

cAMP was determined by radioimmunoassay using the method of Lin et al. (1985). Briefly, the procedure was as follows. Samples or standards (5 to 2000 fmol/tube) in 25 to 100 μl were made up to 200 μl with 50 mM acetate buffer, pH 6.2, and acetylated with 5 μl acetic anhydride : triethylamine (1 : 2, v/v). After 15 min at room temperature, [^{125}I]succinyl cAMP antiserum, sufficient to bind 30–60% of the radioactive ligand, was added. After 4 h at room temperature, rabbit serum carrier and the second antiserum (anti-rabbit Ig from sheep, goat, or burro, Meloy Labs) were added. After standing overnight at 4°C, 2 ml of cold 10 mM acetate buffer, pH 6.2, was added, the tubes were centrifuged, and the radioactivity in the pellets counted. Each sample was measured in duplicate.

2.3. Statistical analysis

All data are expressed as mean ± S.E.M. Analysis of variance, Bonferroni's inequality, and Student's *t*-test were used for comparison of groups. Curve fitting was determined using the Inplot program (GraphPad, San Diego, CA).

2.4. Chemicals

Corticotropin-releasing factor (human/rat) and α-helical CRF-(9–41) were purchased from Peninsula Laboratories (Belmont, CA). Other chemicals used in this study were fura-2/AM, pluronic acid F-127, 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8, Molecular Probes, Eugene, OR), benzamil (Research Biochemicals, Natick, MA), amiloride, verapamil HCl, $LaCl_3$, $CoCl_2$, pertussis toxin, suramin, (+)-*N*-methylglucamine, ryanodine, IBMX (Sigma Chemicals, St. Louis, MO). 1-{6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-1*H*-tyrrole-2,5-dione (U-73122) was provided by Upjohn Co.(Kalamazoo, MI). Naloxone was a gift from Dr. Brian Cox.

3. Results

3.1. Effect of CRF on $[Ca^{2+}]_i$

The resting $[Ca^{2+}]_i$ in adherent cells at 37°C in normal Na^+ Hanks' solution was 66 ± 4 nM (n = 32). When cells were treated with CRF, there was an immediate increase (within 10 s) in $[Ca^{2+}]_i$ dependent on the concentration of CRF (Fig. 1A). The median effective concentration (EC_{50}) of CRF was 11 pM. A concentration of 42 pM induced a maximal increase in $[Ca^{2+}]_i$ (237 ± 20% control, n = 10) (Fig. 1B). The increase induced by CRF was dependent on the presence of external Ca^{2+}. In Ca^{2+}-free buffer containing 10 mM EGTA, the CRF-induced increase in $[Ca^{2+}]_i$ was not observed (Figs. 2 and 3). When increasing concentrations of Ca^{2+} were added back to the buffer, $[Ca^{2+}]_i$ increased proportionally, suggesting that the CRF-induced increase in $[Ca^{2+}]_i$ is a result of Ca^{2+} entry from external sources. When cells were incubated in a buffer without Na^+ (replaced with equimolar (+)-*N*-methylglucamine) or Mg^{2+}, the resting $[Ca^{2+}]_i$ increased significantly. Removal of external Na^+ activates the

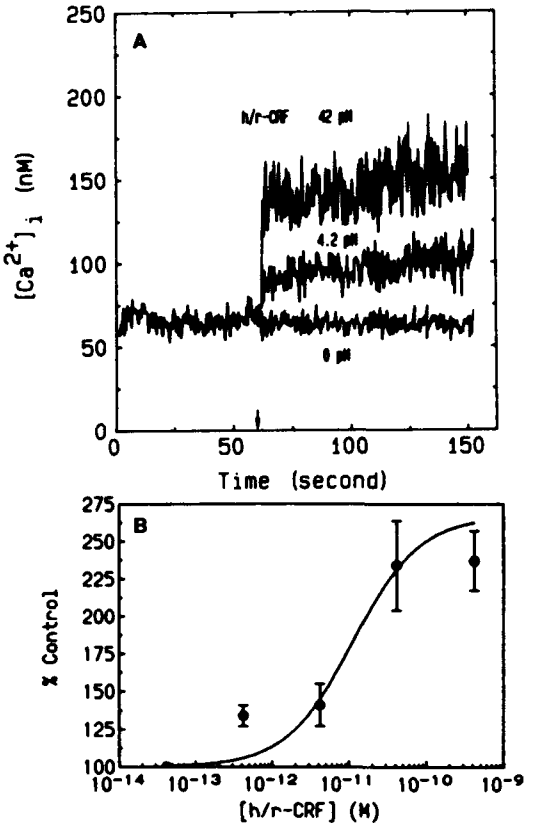

Fig. 1. CRF increases $[Ca^{2+}]_i$. Cells were treated with CRF at different concentrations (n = 3–4). The fluorometer tracing (A) and a sigmoid-curved fit (B) are presented. The calculated median effective concentration was 11 pM.

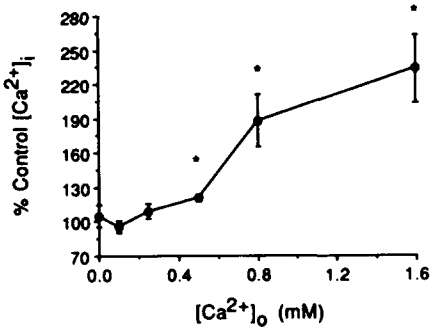

Fig. 2. Effect of external Ca^{2+} ($[Ca^{2+}]_o$) on the CRF-induced increase in $[Ca^{2+}]_i$. Cells were exposed to 42 pM CRF in Na^+ Hanks' solution containing different $[Ca^{2+}]$ (n = 3–6). * $P < 0.05$ vs. 0 external Ca^{2+}.

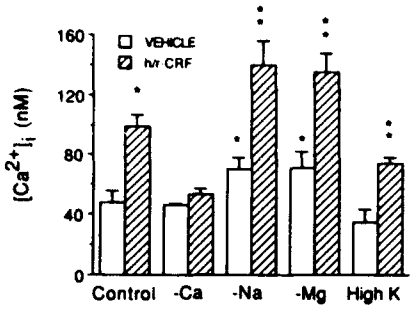

Fig. 3. Effect of external ions on CRF-induced increase in $[Ca^{2+}]_i$. The control buffer contained in mM 145 NaCl, 4.5 KCl, 1.3 $MgCl_2$, 1.6 $CaCl_2$, and 5 HEPES. The Ca^{2+}-free buffer contained 10 mM EGTA without Ca^{2+}. The Na^+-free buffer contained 145 mM *N*-methylglucamine to substitute Na^+. The Mg^{2+}-free buffer contained no Mg^{2+}. The high K^+ buffer contained 25 mM K^+. Cells were placed in each buffer for 5 min before treatment with CRF (42 pM, $n = 3$–5). * $P < 0.05$ vs. vehicle control, ** $P < 0.05$ vs. respective vehicle, two-way ANOVA, and Bonferroni's inequality.

Na^+/Ca^{2+} exchanger (Kiang et al., 1992), leading to an increase in the resting $[Ca^{2+}]_i$. The reason for the increase resulting from the removal of Mg^{2+} is not clear. However, CRF still induced the same magnitude of $[Ca^{2+}]_i$ increase in cells in absence of either cations (Fig. 3).

It has been reported that membrane depolarization can increase $[Ca^{2+}]_i$ in some cells (Hagiwara, 1983). I therefore sought to determine if the increase in $[Ca^{2+}]_i$ is caused by a CRF-induced depolarization of the A-431 cell membrane. Cells were first bathed in a buffer containing 25 mM K^+ (normal $[K^+] = 4.5$ mM), conditions that will depolarize the membrane of sensitive cells. However, this treatment did not change the resting $[Ca^{2+}]_i$, and CRF induced the same magnitude of $[Ca^{2+}]_i$ increase as that measured in cells incubated with normal buffers (Fig. 3). These results indicate that the increase in $[Ca^{2+}]_i$ stimulated by CRF is not a result of a membrane depolarization.

3.2. Effect of Ca^{2+} channel blockers

We sought to determine the role of extracellular Ca^{2+} in the increase in $[Ca^{2+}]_i$ stimulated by CRF. The entry of Ca^{2+} into cells can be blocked by Co^{2+} or La^{3+} (inorganic Ca^{2+} channel blockers) in the external medium (Hagiwara, 1983). Table 1 shows that treatment with either Co^{2+} or La^{3+} slightly changed the resting $[Ca^{2+}]_i$, but CRF did not increase $[Ca^{2+}]_i$ in the presence of either blocker. These results reinforce the concept that the increase in $[Ca^{2+}]_i$ induced by CRF is due to a Ca^{2+} influx.

Different results were obtained with the organic Ca^{2+} channel blockers, verapamil (10 μM) and nifedipine (10 μM). Although these agents by themselves induced a change in the resting $[Ca^{2+}]_i$, treatment with CRF in the presence of either verapamil or nifedipine stimulated a significant additional increase in $[Ca^{2+}]_i$ (Table 1). Much higher concentrations of verapamil and nifedipine (up to 1 mM) also failed to inhibit the CRF-induced increase in $[Ca^{2+}]_i$ (data not shown). These results indicate that the Ca^{2+} entry stimulated by CRF does not occur through voltage-gated Ca^{2+} channels. The data are consistent with the view that A-431 cells are not excitable (Kiang et al., 1992, Moolenaar et al., 1986) and that the $[Ca^{2+}]_i$ increase is not associated with membrane depolarization.

Because Co^{2+} and La^{3+} can function not only to block Ca^{2+} channels but also to block Na^+/Ca^{2+} exchange (Trosper and Philipson, 1983; Kaczorowski et al., 1989), it was necessary also to assess the effect of CRF on the Na^+/Ca^{2+} exchanger. This antiporter system located at the cell membrane translocates Ca^{2+}, coupled to the movement of Na^+ in the opposite direction. The direction of ion transport across the plasma membrane depends upon the prevailing ionic gradients established by the Na^+/K^+-ATPase, the Na^+/H^+ antiporter, and Na^+ channels. Cells were treated with 1 mM amiloride or 100 μM benzamil, inhibitors of the Na^+/Ca^{2+} exchanger. Table 2 shows that amiloride and benzamil both increased the resting $[Ca^{2+}]_i$, indicating that a Na^+/Ca^{2+} exchanger is present in these cells. However, CRF treatment still increased $[Ca^{2+}]_i$ in amiloride- or benzamil-treated cells, excluding the possibility that the Na^+/Ca^{2+} exchange system is inhibited by CRF.

3.3. Effect of α-helical CRF-(9–41)

The increase in $[Ca^{2+}]_i$ by CRF was inhibited by α-helical CRF-(9–41), a CRF receptor antagonist (Rivier et al., 1984). The degree of inhibition depended on the concentration of the inhibitor (Fig. 4A), with the median inhibitory concentration (IC_{50}) occurring at

Table 1
Inorganic Ca^{2+} channel blockers inhibited the CRF-induced increase in $[Ca^{2+}]_i$

	$[Ca^{2+}]_i$ (nM)	
	Control	CRF
-	57 ± 9	150 ± 20 [a]
La^{3+}	63 ± 4	63 ± 5
Co^{2+}	21 ± 6 [a]	21 ± 7 [a]
Verapamil	82 ± 27 [a]	215 ± 84 [b]
Nifedipine	99 ± 16 [a]	209 ± 31 [b]

Cells were treated with Ca^{2+} channel blockers 10 μM for 1 min before 42 pM CRF ($n = 5$).
[a] $P < 0.05$ vs. controls without treatment with Ca^{2+} channel blockers.
[b] $P < 0.05$ vs. respective control, two-way ANOVA, and Bonferroni's inequality.

Table 2
Amiloride, benzamil, U-73122, TMB-8, and ryanodine did not inhibit the CRF-induced increase in $[Ca^{2+}]_i$

	Concentration	$[Ca^{2+}]_i$ (nM)	
		Control	CRF
-	-	57 ± 9	150 ± 20 [a]
Amiloride	1 mM	115 ± 10 [a]	226 ± 9 [b]
Benzamil	100 μM	82 ± 5 [a]	167 ± 14 [b]
U-73122	5 μM	173 ± 12 [a]	264 ± 12 [b]
TMB-8	100 μM	114 ± 18 [a]	283 ± 55 [b]
Ryanodine	100 μM	35 ± 7	104 ± 16 [b]

Cells were treated with amiloride, benzamil, U-73122, TMB-8, or ryanodine 1 min prior to CRF (42 pM) ($n = 4-5$).
[a] $P < 0.05$ vs. control without any treatment,
[b] $P < 0.05$ vs. respective control, two-way ANOVA, and Bonferroni's inequality.

33 nM and complete inhibition at 1 μM (Fig. 4B). These results suggest that the CRF-induced increase in $[Ca^{2+}]_i$ is mediated by receptor-operated Ca^{2+} channels.

If the increase in $[Ca^{2+}]_i$ induced by CRF were mediated by a receptor that coupled to G proteins to open Ca^{2+} channels, treatment with pertussis toxin would also block the increase, because pertussis toxin has been shown to block Ca^{2+} channels that are stimulated by receptor-linked G_k/G_o proteins (Gilman, 1987; Lyengar and Birnbaumer, 1987; Spiegel et al., 1988). On the other hand, if increases in $[Ca^{2+}]_i$ induced by CRF were directly mediated by a receptor coupled to Ca^{2+} channels, then treatment with pertussis toxin should not inhibit the increase. Cells incubated with 30 ng/ml pertussis toxin for 24 h demonstrated an increase in $[Ca^{2+}]_i$ of only 38 ± 8% ($n = 5$, $P < 0.05$, Student t-test) after treatment with 42 pM CRF, whereas CRF increased $[Ca^{2+}]_i$ 134 ± 20% ($n = 10$, $P < 0.05$, Student's t-test) in the absence of pertussis toxin treatment, suggesting that the CRF receptor is coupled to a pertussis toxin-sensitive G protein.

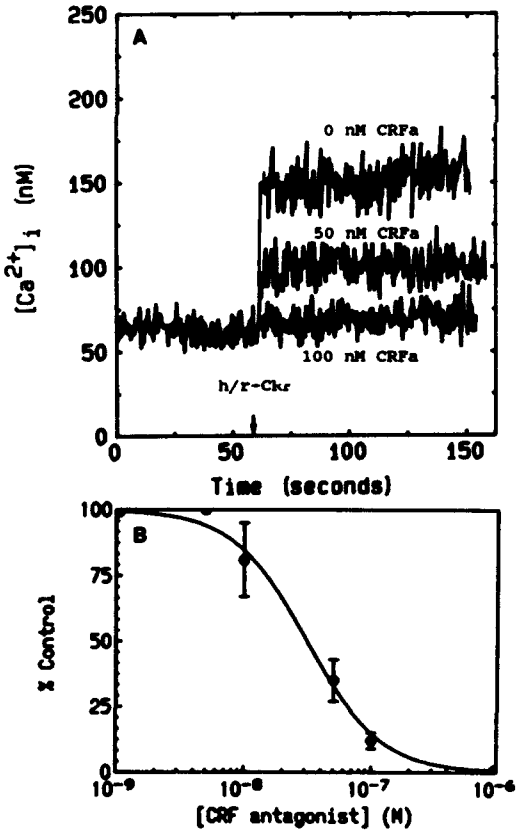

Fig. 4. Inhibition of the CRF-induced increase in $[Ca^{2+}]_i$ by CRF antagonist. Cells were treated with CRF antagonist (CRFa) at the indicated concentrations 1 min prior to challenge with 420 pM CRF ($n = 5$). The fluorometer tracings (A) and the sigmoid-curved fit (B) are presented. The calculated median inhibitory concentration was 33 nM.

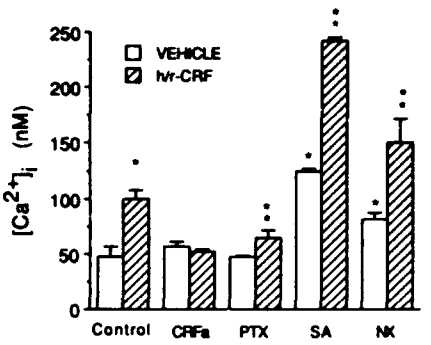

Fig. 5. Effect of CRF antagonist, pertussis toxin, suramin, and naloxone on the CRF-induced increase in $[Ca^{2+}]_i$. Cells were treated with CRF antagonist (CRFa, 100 nM), suramin (SA, 100 μg/ml), naloxone (NX, 50 μM) for 1 min, or pertussis toxin (PTX, 30 ng/ml) for 24 h before challenging with 42 pM CRF ($n = 3-5$). * $P < 0.05$ vs. vehicle control, ** $P < 0.05$ vs. respective vehicle, two-way ANOVA, and Bonferroni's inequality.

The inhibition by α-helical CRF-(9–41) was specific because suramin (a purinergic receptor inhibitor) and naloxone (an opiate receptor antagonist) did not inhibit the increase in $[Ca^{2+}]_i$ induced by CRF (Fig. 5). (Treatment of cells with either suramin or naloxone in the absence of CRF increased the resting $[Ca^{2+}]_i$, but the reason for this increase was not understood.) I sought to determine whether α-helicle CRF-(9–41) interfered with agents other than CRF that can stimulate an increase in $[Ca^{2+}]_i$. ATP is an agent that has been shown to increase Ca^{2+} influx in A-431 cells (Gonzales et al., 1989). ATP (100 μM) increased $[Ca^{2+}]_i$ by 303 ± 43% ($n = 5$) in the absence of α-helical CRF-(9–41). In the presence of α-helical CRF-(9–41), ATP still increased $[Ca^{2+}]_i$ by 232 ± 56% ($n = 4$), which was not significantly different from the increase observed in the absence of α-helical CRF-(9–41) ($P > 0.05$, Student's t-test). The results suggest that the CRF-induced increase in $[Ca^{2+}]_i$ is specifically mediated by CRF receptors.

3.4. Effect of CRF on cAMP

CRF at 42 pM for 10 min induced a maximal increase in $[Ca^{2+}]_i$. However, this concentration did not increase cellular cAMP content either in the presence or absence of external Ca^{2+} in the first 10 sec when the increase in $[Ca^{2+}]_i$ was observed. This would exclude the involvement of second messenger-operated Ca^{2+} channels activated by cAMP. It is important to note that a 10 min incubation with CRF increased cellular cAMP from 1.0 ± 0.2 pmole/10^6 cells to 1.4 ± 0.1 pmole/10^6 cells ($n = 3$, $P < 0.05$, Student's t-test). Any future study of the prolonged effect of CRF should therefore consider the involvement of cAMP in any observed increases in $[Ca^{2+}]_i$, because a 10 min treatment with 1 mM 8-bromo-cAMP in A-431 cells is capable of increasing $[Ca^{2+}]_i$ by $35 \pm 5\%$ (Kiang and McClain, 1993).

3.5. Effect of CRF on intracellular Ca^{2+} stores

Three intracellular Ca^{2+} pools have been indentified in A-431 cells: $InsP_3$-, monensin-, and ionomycin-sensitive pools (Kiang and Smallridge, unpublished data). My data indicate that the $InsP_3$-sensitive Ca^{2+} pool is not involved in the CRF response, because CRF did not increase $InsP_3$ within the same time frame (10 s) as that in which the increase in $[Ca^{2+}]_i$ was observed. An increase in $InsP_3$ was observed only after a 10 min treatment with CRF (from $0.37 \pm 0.06\%$ total cpm to $0.57 \pm 0.07\%$ total cpm, $n = 3$ for both groups, $P < 0.05$, Student's t-test). Furthermore, an inhibitor of $InsP_3$ production, U-73122, did not inhibit the increase in $[Ca^{2+}]_i$ induced by CRF (Table 2). Two inhibitors of intracellular Ca^{2+} mobilization, TMB-8 (a general block for Ca^{2+} stores, Chiou and Malagodi, 1975; Mix et al., 1984; McCoy et al., 1988) or ryanodine (a blocker for non-$InsP_3$-sensitive Ca^{2+} stores, see review Tsien, 1990) were tested to determine whether other intracellular pools were associated with the increase in $[Ca^{2+}]_i$. Treatment with TMB-8 (100 μM) or ryanodine (100 μM) should not interfere with an increase in $[Ca^{2+}]_i$ induced by CRF if the increase were derived primarily from external sources. That was indeed the case. Neither TMB-8 nor ryanodine blocked the increase, which indicates that intracellular Ca^{2+} stores are not involved in the CRF-induced increase in $[Ca^{2+}]_i$.

4. Discussion

The resting $[Ca^{2+}]_i$ in adherent A-431 cells was 66 ± 4 nM. CRF induced an immediate concentration-dependent increase in $[Ca^{2+}]_i$ with an EC_{50} of 11 pM. The increase was apparently due to an influx of extracellular Ca^{2+} because (1) the increase did not occur if Ca^{2+} was omitted from the buffer, and (2) inorganic Ca^{2+} channel blockers inhibited the increase. Voltage-gated Ca^{2+} channels are not involved because organic Ca^{2+} channel blockers such as verapamil and nifedipine failed to block the increase. This is in agreement with the results of Moolenaar et al.(1986) and this laboratory (Kiang, 1991; Kiang et al., 1992). Changes in resting $[Ca^{2+}]_i$ in cells treated with Ca^{2+} channel blockers are probably due to their ability to affect H^+-sensitive Ca^{2+} channels (Kiang, 1991).

The increase in $[Ca^{2+}]_i$ induced by CRF was inhibited by the CRF receptor antagonist, α-helical CRF-(9–41). The IC_{50} required to block the activity of CRF (420 pM) was 33 nM. The inhibition was specific because neither suramin nor naloxone inhibited the CRF-induced increase in $[Ca^{2+}]_i$ and because this CRF receptor antagonist failed to inhibit the increase in $[Ca^{2+}]_i$ induced by ATP. Treatment with pertussis toxin attenuated the $[Ca^{2+}]_i$ response to CRF. These results taken together suggest that the Ca^{2+} channels involved with CRF activity are CRF receptor-operated and coupled to a pertussis toxin-sensitive G protein.

Because Ca^{2+} influx can be mediated by second messenger-operated Ca^{2+} channels, increases in $[Ca^{2+}]_i$ may result from CRF-stimulated increases in cellular cAMP. Previously this laboratory showed that an exogenous application of 1 mM 8-bromo-cAMP did not immediately change resting $[Ca^{2+}]_i$ in A-431 cells (Kiang et al., 1991), although prolonged incubation with 8-bromo-cAMP led to $[Ca^{2+}]_i$ increases (Rinaldi et al., 1981; Kiang and McClain, 1993). In the present study, cAMP did not increase during the same period that $[Ca^{2+}]_i$ increased after CRF treatment. That is, the $[Ca^{2+}]_i$ increase was immediate, whereas cAMP failed to increase until 10 min., suggesting that second-messenger operated Ca^{2+} channels are not stimulated by CRF.

Intracellular Ca^{2+} pools also do not appear to be involved in the CRF-induced increase in $[Ca^{2+}]_i$. This view is supported by two observations. First, $InsP_3$ did not increase during the same period that $[Ca^{2+}]_i$ increased after CRF treatment. Second, U-73122 (an inhibitor for $InsP_3$ production), TMB-8 or ryanodine (inhibitors of intracellular Ca^{2+} mobilization) failed to inhibit the increase.

Polyvalent cations have been reported to block Na^+/Ca^{2+} exchange (Trosper and Philipson, 1983; Kaczorowski et al., 1989). Such was not the case here because amiloride and benzamil, inhibitors of the Na^+/Ca^{2+} exchanger, did not inhibit the increase in $[Ca^{2+}]_i$. This is supported further by the observation that removal of external Na^+ did not block the CRF effect on $[Ca^{2+}]_i$.

The physiological significance of changes in $[Ca^{2+}]_i$ has been documented in pituitary cells and in hip-

pocampus. It is highly likely that an increase in $[Ca^{2+}]_i$ stimulated by CRF is a common signal for various properties possessed by CRF. In mouse pituitary AtT-20 cells, the CRF-induced increase in c-*fos* mRNA is Ca^{2+}-dependent (Boutillier et al., 1991). In dispersed rat anterior pituitary cells 10 nM ovine CRF increased ACTH release. When $[Ca^{2+}]_i$ was lowered, ACTH release was attenuated (Won and Orth, 1990). This is probably due to the ability of CRF to increase $[Ca^{2+}]_i$ (Guerineau et al., 1991). In vivo studies show that CRF injected into the dentate gyrus of the hippocampus enhances memory. Nifedipine and verapamil both antagonize the memory-enhancing effect of CRF (Lee and Lin, 1991). In this study, CRF at concentrations between a normal level and a pregnant level induced a moderate increase in $[Ca^{2+}]_i$ in A-431 cells that was mediated by CRF-receptor operated Ca^{2+} channels. The apparent contradiction that nifedipine and verapamil antagonized the memory-enhancing effect of CRF in rat's brain (Lee and Lin, 1991) but did not inhibit the CRF-induced increase in $[Ca^{2+}]_i$ in A-431 cells can be attributed to the different types of cells used in these experiments.

In summary, this paper demonstrates that CRF increased $[Ca^{2+}]_i$ in nonendocrine cells in a concentration-dependent manner. The increase was blocked by removal of external Ca^{2+}, treatment with polyvalent cations, pertussis toxin, or treatment with a CRF antagonist. The data suggest that the increase is due to a Ca^{2+} influx through CRF-receptor mediated Ca^{2+} channels.

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